Development and validation of a quantitative confirmatory method for 30 β-lactam antibiotics in bovine muscle using liquid chromatography coupled to tandem mass spectrometry

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Keywords: β-Lactam residue; Penicillin; Cephalosporin; Carbapenem; UHPLC-MS/MS; Bovine muscle

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Abstract

A method was developed for the confirmatory and quantitative analysis of 30 β-lactam antibiotic residues in bovine muscle. The method includes 12 penicillins (amoxicillin, ampicillin, cloxacillin, dicloxacillin, mecillinam, methicillin, nafcillin, oxacillin, penicillin G, penicillin V, piperacillin, ticarcillin), 12 cephalosporins (cefacetrile, cefadroxil, cephalexin, cefalonium, cefazolin, cefoperazone, cefotaxime, cefquinome, cefuroxime, desacetyl cephapirin, desfuroylceftiofur cysteine disulfide, desfuroylceftiofur dimer), five carbapenems (biapenem, doripenem, ertapenem, imipenem, meropenem) and faropenem. Samples were

extracted using a simple solvent extraction with acetonitrile:water (80:20, v/v) and C_{18} dispersive solid-phase extraction (d-SPE) clean-up, followed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) detection. Chromatography was performed on a reversed phase CSH C_{18} column, using a binary gradient separation comprising of 0.01% formic acid and 0.2 mM ammonium acetate in water (mobile phase A) and 0.01% formic acid in acetonitrile (mobile phase B). The mass spectrometer was operated in the positive electrospray ionisation mode (ESI(+)). Validation was performed following the 2002/657/EC guidelines. Trueness ranged between 69% and 143% and precision ranged between 2.0% and 29.9% under within-laboratory reproducibility conditions. Absolute recoveries ranged from 40.3% to 89%. The developed method uses minimal sample preparation and 30 test samples can be analysed by a single analyst in a single day. To the best of our knowledge, this is the first method for carbapenems in foodstuff that does not require derivatisation.

1. Introduction

The β-lactams are key antibiotics used in both human and veterinary medicine [1]. All of these drugs have a common four-membered ring in their molecular structure (Fig. 1). The penicillins, cephalosporins and carbapenems represent the most important β-lactam drug groups. In the penicillin and cephalosporin structures, the four-membered ring is fused to a five-membered thiazolidine or a six-membered dihydrothiazine ring, respectively. The carbapenems are similar to the penicillins, with a sulphur atom replaced by a carbon atom. Faropenem, a carbapenem-related compound, is another important member of the β-lactam class [2].

The β-lactams act by inhibiting the enzymes (PBPs, Penicillin-Binding Proteins) involved in the biosynthesis of the peptidoglycan, a fundamental constituent of the bacterial cell wall [3]. This is particularly important for actively multiplying bacteria, in which the β-lactams can cause cell lysis. Penicillins are classified as broad- or narrow-spectrum antimicrobials, based on their activity, or according to their susceptibility to β-lactamases produced by bacteria [1]. Cephalosporins are less susceptible to the action of β-lactamases and can be categorised into different generations [4]. In particular, their activity against Gram-positive bacteria decreases between the first and third generations, while their effectiveness against Gram-negative bacteria increases. The fourth-generation cephalosporins are broad-spectrum antimicrobials that are active against both Gram-positive and Gram-negative organisms [1].

Penicillins and cephalosporins are often administered parenterally and orally to food-producing animals to prevent or treat bacterial infections. An inappropriate use of antibiotics may lead to residues in food, which can cause health hazards, such as allergic reactions in sensitive individuals. In addition, concerns have been raised over the excessive usage of antibiotics, which is linked to the increase of antimicrobial resistance in livestock and the potential transfer of resistant bacteria and resistant genes to humans and human pathogens, respectively [3]. This is particularly important among the third- and fourth-generation cephalosporins, due to the reliance of these drugs in human medicine as broad-spectrum antibiotics [5, 6]. Consequently, Maximum Residue Limits (MRLs) are established for different species and target tissues [7] and accurate methods become essential to measure the presence of non-compliant antibiotic residues in food.

The monitoring of β-lactams in food of animal origin is complicated because of the differences in authorisation in food producing species. There are nine penicillin and eight

cephalosporin active ingredients listed under Commission Regulation (EU) 37/2010 [7]. Many of the penicillin drugs are licensed for use in multiple species. For example, amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin and penicillin G are allowed in the treatment of all food-producing species. The other penicillins are licensed more specifically, e.g. nafcillin (all ruminant species), penicillin V (poultry and swines) and mecillinam (cattle only).

The usage of cephalosporin antibiotics in food production has attracted much attention in recent years with calls to restrict the applications of third- and fourth-generation drugs. The European Commission recommends that the use of a narrow-spectrum antimicrobial should always be the first choice unless prior susceptibility testing shows that this would be ineffective [8]. Consequently, third- and fourth-generation cephalosporins should only be administered systemically to animals when clinical conditions respond poorly to narrow spectrum antibiotics. Among the first-generation cephalosporins, cefacetrile, cephalexin, cefalonium and cephapirin are approved for the treatment of cattle, while cefazolin is approved for cattle, goats and swines. Cefoperazone and ceftiofur belong to the third generation and are allowed in the treatment of cattle and all mammalian food-producing species, respectively. Cefquinome, a fourth-generation cephalosporin, is approved for cattle, horses and swine [7]. The carbapenems are broad spectrum antibiotics, resistant to most β-lactamases. These drugs are not registered for use in food-producing animals, but play a critically important role in human medicine [9]. As a consequence, analytical methodologies should be capable of detecting MRL breaches for licensed drugs and the presence of residues from unauthorised applications.

Different screening approaches are available for detecting antibiotic residues in food samples (immunoassays, microbial inhibition assays, receptor assays). The main advantages of these tests are simplicity, low cost and short analysis time, with no need for laborious sample preparation. However, most of these methods cannot identify or quantify the antibiotic that is present. In addition, some of these tests can lead to false positive or false negative results [10]. Liquid chromatography coupled to mass spectrometric detection has become the preferred technique to quantify and confirm the presence of β-lactams in foodstuffs. Several LC-MS(/MS) methods have been reported for the analysis of β-lactams in food matrices, mostly for milk [11-14]. A number of methods have also been published for the analysis of β-lactams in tissue, including kidney [15-25], liver [20, 21, 24] and muscle [19-21, 23, 25-40]. The majority of these methods include a few penicillins and cephalosporins, or apply to specific drugs. The most comprehensive method was reported by Berendsen *et al.* for 22 β-lactams in poultry muscle, including penicillins, cephalosporins and carbapenems [2]. Becker *et al.* also developed a sensitive and specific method for 15 β-lactams in bovine milk, muscle and kidney [41]. However, in both cases, the sample preparation involves a laborious procedure with solid phase extraction (SPE) clean-up.

The objective of this study was to advance this analytical area and develop a comprehensive UHPLC-MS/MS method for monitoring the presence of the major β-lactams in both domestic and imported foods. The method aims to cover the antibiotics permitted for use in food-producing animals as listed under EU legislation [7], novel drugs that are considered as critically important antibiotics for human use only, and metabolites, such as desacetyl cephapirin (DAC), desfuroylceftiofur cysteine disulfide (DCCD) and desfuroylceftiofur dimer (DCD). A further objective of this study was to develop a fast and easy sample

preparation procedure that will reduce sample analysis time and simplify method implementation in regulatory laboratories.

2. Materials and Methods

2.1. Chemicals, standards and consumables

Ampicillin trihydrate, cloxacillin sodium salt monohydrate, dicloxacillin sodium salt hydrate, mecillinam, methicillin sodium salt, nafcillin sodium salt monohydrate, oxacillin sodium salt monohydrate, penicillin V potassium salt, cefalonium hydrate, cephapirin sodium, biapenem, doripenem monohydrate, meropenem trihydrate and faropenem sodium hydrate were purchased from Sigma-Aldrich (Dublin, Ireland). Amoxicillin trihydrate, penicillin G potassium salt, piperacillin, ticarcillin monosodium, cefadroxil hydrate, cefazolin sodium salt, cefoperazone sodium salt, cefotaxime sodium salt, cefquinome sulphate, cefuroxime sodium salt, cephalexin monohydrate and imipenem were purchased from LGC Standards (Teddington, Middlesex, UK). DCCD and DCD were a gift from Zoetis (Kalamazoo, MI, USA). Cefacetrile, DAC sodium salt, ertapenem disodium 90% and the 10 internal standards amoxicillin-D_4, ampicillin-D_5, benzyl penicillanate-D_7 potassium salt (penicillin G-D_7), nafcillin-D_5 sodium salt, penicillin V-D_5, cefadroxil-D_4 (major), cefazolin-$^{13}C_2{}^{15}N$ sodium salt, cephalexin-D_5 hydrate, DAC-D_6 sodium salt (major) and DCCD-D_3 were supplied by Toronto Research Chemicals (Toronto, ON, Canada).

Ultra-pure water (18.2 MΩ cm^{-1}) was generated in-house using a Millipore water purification system (Millipore, Cork, Ireland). Acetonitrile (MeCN), methanol (MeOH) and hexane (all UHPLC grade) were supplied by Romil Ltd (Cambridge, UK). Dimethyl sulfoxide (DMSO),

149 formic acid (HCOOH) 98-100%, trifluoroacetic acid (TFA), ammonium formate and
150 ammonium acetate were supplied by Sigma-Aldrich. Glacial acetic acid (CH_3COOH) 100%
151 was purchased from Merck (Darmstadt, Germany). Sorbents tested for d-SPE included NH_2
152 (Bondesil-NH_2 bulk sorbent, Agilent Technologies), PSA (Supelclean^{TM} primary/secondary
153 amine SPE bulk sorbent, Sigma-Aldrich), C_8 ISOLUTE bulk sorbent (Biotage, Uppsala,
154 Sweeden), C_{18} (Agilent Technologies Ltd, Cork, Ireland), Z-Sep, Z-Sep+ and Z-Sep/C_{18}
155 (Supel^{TM} QuE, 15 mL tubes, Sigma-Aldrich). Anhydrous magnesium sulphate ($MgSO_4$) and
156 sodium chloride (NaCl) were sourced from Sigma–Aldrich and Applichem (Darmstadt,
157 Germany), respectively, and used as part of the QuEChERS (Quick, Easy, Cheap, Effective,
158 Rugged and Safe) approach.
159
160 Polypropylene tubes (15 mL and 50 mL) were obtained from Sarstedt Ltd (Wexford, Ireland).
161 Membrane filters (Captiva Econo Filter PTFE 13m 0.2 µm) were sourced from Agilent
162 Technologies Ltd. A ME36S microbalance and an A200S digital electronic analytical
163 balance, both from Sartorius (Dublin, Ireland), were used for standard preparation (weighing
164 of standard material and required solvent, respectively). An Ultra-Turrax probe blender from
165 IKA (Staufen, Germany), a MSE Mistral 3000i centrifuge from Davidson and Hardy (Dublin,
166 Ireland) and a TurboVap LV evaporator from Biotage were used for sample preparation.
167
168 *2.2. Preparation of standard solutions*
169 Individual stock solutions of ampicillin, cloxacillin, dicloxacillin, mecillinam, methicillin,
170 nafcillin, oxacillin, penicillin G, penicillin V, ticarcillin, cefadroxil, cephalexin, cephapirin,
171 cefazolin, cefoperazone, cefotaxime, cefuroxime, biapenem, doripenem, ertapenem,
172 imipenem, meropenem, faropenem and the internal standards ampicillin-D_5, penicillin G-D_7,
173 penicillin V-D_5, nafcillin-D_5, cefadroxil-D_4, cefazolin-$^{13}C_2{}^{15}N$ and cephalexin-D_5 were

prepared at a concentration of 1 mg mL^{-1} in H_2O:MeCN (75:25, v/v). Stock solutions of piperacillin and cefalonium were prepared at a concentration of 1 mg mL^{-1} in H_2O:MeCN (50:50, v/v). Amoxicillin, amoxicillin-D_4, DCCD and DCCD-D_3 were dissolved in H_2O:MeCN (50:50, v/v) at a concentration of 0.5 mg mL^{-1}. Cefacetrile, cefquinome, DAC, DAC-D_6 and DCD were prepared in DMSO at a concentration of 1 mg mL^{-1}. After preparation, the solutions were stored in 2.5 mL aliquots in 15 mL polypropylene tubes at -80°C. Under these storage conditions, individual stock solutions were found to be stable for at least two months. Three intermediate standard solutions containing all the β-lactams at concentrations ranging from 4 µg mL^{-1} to 200 µg mL^{-1} were prepared in water. Working calibration solutions were prepared by diluting the intermediate stock solutions in water. A mixed internal standard solution was prepared at concentrations ranging from 0.2 µg mL^{-1} to 10 µg mL^{-1} in water. Working solutions were found to be stable for at least one month when stored at -80°C.

2.3. Sample preparation

Bovine muscle homogenate samples not containing any detectable β-lactam residues were used as negative controls. Sample aliquots (2 g ± 0.01 g) were weighed into a 50 mL polypropylene centrifuge tube. Extracted matrix calibrants were fortified with 100 µL of the working standard solutions. A 100 µL volume of internal standard solution was added to all calibrants, controls and test samples, which were then allowed to stand for 15 min. Water (1.9 mL for the extracted matrix calibrants and 2 mL for the controls and test samples) and MeCN (8 mL) were added in order to extract the analytes from the matrix, and the polypropylene tubes containing the samples were subsequently homogenised for 20 s over ice using an Ultra-Turrax probe blender. The homogenised samples were centrifuged for 15 min at 2842×*g* (4°C) and the supernatant was decanted into a 50 mL polypropylene tube containing

500 mg of C_{18} sorbent. The samples were vortexed (40 s) and centrifuged for 15 min at 2842×*g* (4°C). A 5 mL aliquot of the supernatant was placed into a 15 mL polypropylene tube and evaporated under nitrogen on a TurboVap at 40°C to a final aqueous volume of < 1 mL. Solvent evaporation conditions required careful control to avoid degradation or loss of analytes. An evaporation temperature of 40°C was found to be optimal, while nitrogen gas pressure was dynamically controlled during the 55 min evaporation process. To avoid loss of sample, the nitrogen gas was maintained at 15 psi for the first 20 min. Subsequently, the pressure was gradually increased to 20 psi over 10 min, and the solvent evaporation time was greatly reduced by adapting this approach. After the evaporation, the volume was made up to 1 mL with water and the extracts were vortexed for 10 s prior to filtration through 0.2 µm PTFE syringe filters. The filtered extracts were collected directly into autosampler vials and a 10 µL volume was injected into the UHPLC-MS/MS system.

2.4. Instruments and UHPLC-MS/MS conditions

Samples were analysed using a Waters Acquity UHPLC system coupled to a Waters Quattro Premier triple quadrupole mass spectrometer (Milford MA, USA) equipped with ESI ionisation probe. The UHPLC-MS/MS system was controlled by MassLynx software (V4.1) and the results were processed by TargetLynx software (V4.1). Separation was performed on a stainless steel CSH C_{18} analytical column (2.1 × 100 mm, particle size 1.7 µm) fitted with an in-line filter with a 0.2 µm pore size. The column was maintained at 30°C, as higher temperatures were found to negatively affect the precision of the method. A binary gradient separation comprising of 0.01% HCOOH and 0.2 mM ammonium acetate in H_2O (mobile phase A) and 0.01% HCOOH in MeCN (mobile phase B) was employed at a flow rate of 0.4 mL min^{-1}. The injection volume was 10 µL in full loop mode (overfill factor = 4). The gradient profile was as follows: (1) 0 - 1.5 min, 100% A; (2) 1.5 - 3.5 min, 80% A; (3) 3.5 -

8.5 min, 20% A; (4) 8.5 - 8.6 min, 0% A; (5) 8.6 - 10.5 min, 0% A; (6) 10.5 - 10.6 min, 100% A, which was held for 7.4 min (re-equilibration of the column), for a total run time of 18 min. The UHPLC autosampler was rinsed after each injection using strong (H_2O:MeCN, 20:80 v/v, 750 µL) and weak (0.01% HCOOH in H_2O:MeCN, 90:10 v/v, 1000 µL) washes. Sample temperature was maintained at 7°C in the autosampler. A divert valve was used to reduce source contamination (solvent delay: [a] 0 - 1.97 min; [b] 7.60 - 18 min). The mass spectrometer was operated in positive electrospray ionisation mode (ESI(+)) and the capillary voltage was set at 2.4 kV. Source and desolvation temperatures were 140°C and 450°C, respectively. Nitrogen was used as desolvation and cone gases and set at 1000 L h^{-1} and 100 L h^{-1}, respectively. Argon collision gas flow rate was set at 0.22 mL min^{-1}. The MS tuning was performed by teed infusion of 1 µg mL^{-1} standards/internal standards with mobile phase A:B (50:50, v/v). The cone voltage and collision energy were optimised for each analyte and two product ions were selected so that a minimum of four identification points were obtained for all the analytes as required by Commission Decision 2002/657/EC [42]. A multiple-reaction monitoring (MRM) method was developed with 15 different time-sectored events (Table 1). Inter-scan delay and inter-channel delay were set to 0.005 s.

2.5. Validation

The validation was performed following the 2002/657/EC guidelines [42]. The following parameters were assessed: identification, selectivity, linearity, trueness, within-laboratory repeatability (WLr), within-laboratory reproducibility (WLR), absolute recovery, decision limit (CCα) and detection capability (CCβ). Matrix effects, limit of detection (LOD) and limit of quantitation (LOQ) were also evaluated as part of the validation process.

Identification was assessed by examining retention times, ion ratios and identification points. The selectivity of the method was investigated through injecting standard solutions of all analytes and internal standards individually and through testing 27 bovine muscles from different animals, in order to check the presence of any interferences eluted at the retention times of the analytes. The linearity of the curves was considered satisfactory if $R^2 \geq 0.98$ and if individual residuals did not deviate by more than ± 20% from the calibration curve. For the MRL-substances, trueness, WLr and WLR were assessed at 0.5, 1 and 1.5 times the MRLs established by current legislation [7], except for DCD and DCCD, which were validated at 250, 500 and 750 µg kg^{-1}. The non-MRL substances were validated around a target level (TL) that was identified based on the sensitivity of the method (Table 4). The WLr and WLR studies were both performed on three separate days by three different analysts. To evaluate WLr, a different bovine muscle negative control was used on each day, and eight portions of the sample were fortified at each validation level. To evaluate WLR, 21 different bovine muscle samples were used and seven bovine muscles, plus a mixture of them all, were fortified at each validation level. The available labelled compounds were used in the quantification of their corresponding analytes. Additionally, cloxacillin, dicloxacillin and oxacillin were all corrected using nafcillin-D_5; cefacetrile, cefotaxime, cefuroxime and faropenem were corrected using cefazolin-$^{13}C_2{}^{15}N$; mecillinam and ertapenem using ampicillin-D_5; cefalonium was corrected using cephalexin-D_5 and doripenem using DAC-D_6. No internal standards were used for the other analytes, as the available labelled compounds were found to be unsuitable in their quantification. Absolute recoveries were calculated by comparing results from fortified samples to those of negative samples spiked post-extraction at 2 × lowest calibration level and 0.875 × highest calibration level on three different occasions. For the MRL-substances, CCα and CCβ were calculated from the within-laboratory reproducibility as defined in 2002/657/EC [42]. For the non-MRL substances, the

same approach was used considering the TL, in order to obtain clearly detectable CCα and CCβ values in the range of the calibration curves.

Matrix effects were also evaluated: 27 blank samples were spiked post-extraction at the MRLs or TLs and the signal obtained from those samples was compared to the signal obtained from a standard solution at the same concentration [43]. LODs and LOQs of all analytes were estimated from the blank samples fortified at the lowest calibration level on three different occasions, and measuring the signal-to-noise ratios at 3 and 10, respectively, for both quantifier and qualifier ions.

3. Results and Discussion

3.1. Method development

3.1.1. UHPLC-MS/MS conditions

The MS tuning experiments performed showed protonated molecules $[M+H]^+$ for most of the β-lactams. The monitored precursor ion for DCD was $[M+2H]^{2+}$; faropenem formed the sodium adduct $[M+Na]^+$, while cefacetrile and cefuroxime surprisingly formed the ammonium adduct $[M+NH_4]^+$. For the penicillins and cephalosporins, the product ions selected following low energy collision induced dissociation (CID) experiments generally matched those reported in the literature or characterized by Geis-Asteggiante *et al.* using electrospray ionization and quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) [44]. For the carbapenems, the monitored transitions were in agreement with those reported in the published LC-MS/MS methods for the determination of these drugs in urine, serum and

other biological samples [45-48]. In order to get optimal sensitivity, the following MS parameters were optimised: capillary voltage, cone voltage, collision energy, source temperature and desolvation temperature. The response for mecillinam and nafcillin was very intense, consequently the MS was detuned to avoid saturation of the signal at high concentrations.

The following additives were evaluated at different concentrations in both mobile phases A and B: 0.005%, 0.01% and 0.1% HCOOH; 0.01% and 0.1% CH_3COOH; 0.001% and 0.1% TFA. The addition of ammonium acetate and ammonium formate in mobile phase A only was also investigated at concentrations ranging from 0.1 to 0.5 mM. Also, three different UHPLC column chemistries were assessed, namely, BEH C_{18}, CSH C_{18} and HSS T3. Optimal results were achieved when using a binary gradient separation with a mobile phase containing (A) 0.01% HCOOH and 0.2 mM ammonium acetate in H_2O and (B) 0.01% HCOOH in MeCN, on a CSH C_{18} column. A concentration of 0.01% HCOOH provided the best overall response, while higher concentrations of the same acid or use of different acids decreased sensitivity. Moreover, the removal of TFA from the UHPLC system after its use was found to be very difficult, and its presence seemed to affect other assays performed on the same instrument. The response for all analytes was also decreased when using higher concentrations of salts, consequently 0.2 mM ammonium acetate was added to mobile phase A to promote the formation of the ammonium adduct for two cephalosporins, namely cefacetrile and cefuroxime. The results obtained were in agreement with what reported in the literature. Formic acid is, indeed, the most common acidic agent used in the analysis of β-lactams [11-13]. In addition, Becker *et al.* evaluated the effect of different concentrations of HCOOH and observed that the optimal chromatographic separation and ionisation efficiency were obtained with small amounts of acid in the eluent [41].

Different column temperatures were also tested (30°C, 40°C and 50°C) and the lower temperature was chosen, as it was shown to improve the precision of the method. The analytes particularly affected by the column temperature were imipenem, faropenem, cefuroxime, DCCD and DCD. A series of 25 injections of a 50 ng mL^{-1} mixed standard solution showed that the relative standard deviation (RSD%) for these compounds ranged from 6.3% (cefuroxime) to 11.5% (imipenem) when the column temperature was set at 50°C, while the precision improved at 30°C, with RSDs% ranging from 1.4% (faropenem) to 4.0% (DCD). The autosampler temperature was set at 7°C to prevent a potential degradation of the analytes. During each run performed in the proposed study, a matrix sample fortified at the MRLs/TLs was injected as check sample at evenly spaced intervals throughout the run to monitor the stability of the analytes in the autosampler. All the compounds were found to be stable for at least 24 hours at 7°C, as the absolute area obtained from the check sample stayed constant until the end of the run and the RSD% was not greater than 7.9%, except for imipenem (RSD% = 10.2%), for which the peak area decreased gradually, due to the instability of this compound.

Gradient conditions and flow rate were adjusted in order to reach optimal chromatographic separation. The multiple reaction monitoring chromatograms in Fig. 2 show that all the 30 β-lactams and the 10 internal standards were separated within the first 8 min of the chromatographic run. However, after 8.6 min, the gradient profile was held at 100% B for approximately 2 min, in order to remove the most non-polar matrix components from the column. Subsequently, a 7.4-min hold at 100% A was necessary for column equilibration, resulting in a total cycle time of 18 min.

348 *3.1.2. Sample preparation*

349 One of the main objectives of this study was to develop a rapid and easy sample preparation
350 procedure. The QuEChERS method is well known for being a streamlined approach and was
351 evaluated during preliminary studies, but gave unacceptably low recovery of polar analytes,
352 likely due to partitioning into the aqueous layer. In contrast, a suitable protocol was identified
353 based on the methods developed by Fagerquist *et al.* [17] and Mastovska and Lightfield [22].
354 They found that an extraction solvent composed of MeCN:H_2O (80:20, v/v) was optimal for
355 the isolation of 11 β-lactams from 1 g of bovine kidney. The sample weight was subsequently
356 increased to 2 g by Schneider *et al.* [49] and the extraction procedure was applied to bovine
357 kidney for the analysis of 120 veterinary drugs from different classes, including seven
358 penicillins and eight cephalosporins. Geis-Asteggiante *et al.* [50] also adapted the same
359 sample preparation to 2 g of bovine muscle for monitoring >100 veterinary drug residues,
360 with the inclusion of seven penicillins and three cephalosporins. In our method, the volume of
361 the extraction solvent was kept at 10 mL and the procedure used for the selective and
362 efficient isolation of 12 penicillins, 12 cephalosporins, five carbapenems and faropenem from
363 2 g of bovine muscle samples.

364

365 With the exception of cefazolin, cefotaxime and faropenem, for which no significant
366 difference was observed, all the analytes seemed to be affected by the sample temperature,
367 which needed to be carefully controlled along the sample preparation process to prevent
368 degradation of the β-lactam residues. During the method development, it was found that, after
369 the addition of the extraction solvent, samples required homogenisation over ice, and that the
370 maximum solvent evaporation temperature had to be restricted to 40°C.

371

A number of sorbent materials were investigated for d-SPE: NH_2 (500 mg), PSA (500 mg), C_{18} (500 mg), C_8 (500 mg) and zirconium dioxide-based sorbents (Z-Sep (500 mg); Z-Sep+ (500 mg); Z-Sep/C_{18} (120 mg/ 300 mg)). NH_2 and PSA are sorbents effective at retaining fatty acids and other organic acids present in food. C_{18} is often used for removing lipophilic compounds in food of animal origin, which has high lipid content. C_8 is a sorbent generally employed for the extraction of non-polar compounds from matrices with low-fat content. Z-Sep is a novel bonded silica sorbent that has been developed for the selective isolation of hydrophobic analytes from fatty matrices, while Z-sep/C_{18} or Z-sep+ are recommended for samples containing <15% fat and >15% fat, respectively. A liquid-liquid partitioning clean-up was also tested using 6 mL of hexane, as an alternative to the d-SPE clean-up step. Geis-Asteggiante *et al.* [50] reported a similar study for several veterinary drugs in bovine muscle, including 11 β-lactams. In their work, they evaluated the efficiency of different d-SPE sorbents (C_{18}, Z-Sep, Z-Sep+) and/or partitioning with hexane in removing matrix co-extracting by gravimetric measurements. The liquid-liquid partitioning with hexane was found to be the least efficient, followed by C_{18} clean-up. A combination of Z-Sep and C_{18} resulted in the highest matrix removal, followed by Z-Sep + C_{18} + hexane and Z-Sep alone. However, further investigation on matrix effects and recoveries discouraged the use of these clean-up strategies; consequently, a combination of C_{18} and hexane was used in their sample preparation. In the proposed study, sensitivity, absolute recoveries and precision were evaluated for each of the clean-up approaches tested. NH_2 and PSA sorbents were immediately discarded, as they provided unacceptable results for the majority of the analytes. Mastovska and Lightfield (2008) also found PSA to be unsuitable for the analysis of β-lactams in bovine kidney, finding these drugs were retained by PSA, likely due to the carboxylic group present in the molecular structure [22]. Hexane provided high recoveries for cloxacillin, nafcillin and oxacillin, while the recoveries were not satisfactory for cefuroxime

and DAC when using C_8 sorbent. Among the zirconium dioxide-based sorbents, Z-Sep showed the lowest overall recoveries (particularly for the carbapenems) (Fig. 3). This result was in good agreement with that reported by Geis-Asteggiante *et al.* [50]. In their study, they found that the β-lactams were strongly retained when using a combination of Z-Sep and hexane. This could be due to the fact that zirconium can easily react with the carboxyl and hydroxyl groups present in the β-lactam structures and form strong bonds. In the proposed work, C_{18}, Z-Sep+ and Z-Sep/C_{18} were found to be the most efficient strategies for all the substances, except for imipenem, which was found to be retained also by Z-Sep+ and Z-Sep/C_{18} (Fig. 4). In addition, more precise results were obtained for the majority of compounds when using the C_{18} sorbent. As a consequence, C_{18} was chosen for the proposed sample preparation.

3.2. Standard stability

The stability issues associated with penicillin methanolic stock solutions were discovered when tuning was performed. The standards were initially dissolved in MeOH or H_2O/MeOH and stored at -30°C. The MS tuning of 1 µg mL^{-1} standard aqueous solutions was originally performed immediately after standard preparation and subsequently repeated two weeks later. Interestingly, the MS spectra obtained from the second experiment for cloxacillin, dicloxacillin and penicillin V showed a mass corresponding to their methyl ester $[M+CH_3OH+H]^+$. This was likely due to a breakdown of the β-lactam ring and the formation of a methanolic degradation product. The results raised curiosity, mostly because MeOH is a common solvent used in the analysis of β-lactams. In addition, contradictory information had been found in the literature regarding the stability of penicillin standards in solvent [17, 22, 41, 51-53]. It was decided, then, to investigate the phenomenon for one of the problematic analytes, i.e. cloxacillin. The MS tuning was repeated after preparation of 1 mg mL^{-1} stock

422 solutions in MeOH and H_2O:MeCN (75:25, v/v), which were stored for 11 days in 1 mL
423 aliquots under different storage conditions (room temperature, +4°C, -30°C and -80°C). The
424 spectra obtained from the stock solutions in MeOH showed a mass at 436.0 *m/z* ($[M+H]^+$),
425 but also a mass at 468.0 *m/z* ($[M+CH_3OH+H]^+$) that did not appear in the spectra obtained
426 from the standard dissolved in H_2O:MeCN (Fig. 5). All the different aliquots were
427 subsequently diluted in water at a concentration of 10 µg mL^{-1}. A MS scan was acquired
428 following column injections and the chromatograms extracted for the ions 436.0 and 468.0
429 *m/z*. As shown in Fig. 6, the signal obtained from 436.0 *m/z* was of lower intensity for the
430 standard prepared in MeOH compared to the one prepared in H_2O:MeCN, indicating some
431 degradation for cloxacillin. The degradation in aliquots that had been stored at lower
432 temperature was less (22% degradation at -80ºC and 19% at -30ºC) than the aliquots stored at
433 +4ºC (81% degradation) or room temperature (99% degradation). However, a fresh solution
434 prepared in MeOH also showed a mass at 468.0 *m/z* and 31% degradation for 436.0 *m/z*,
435 which was surprisingly higher than what observed after storage at -80ºC and -30ºC. This was
436 likely due to the fact that the fresh solution had been left at room temperature for
437 approximately three hours before the dilution in water and the column injection. It can be
438 concluded that the degradation of cloxacillin starts as soon as the standard is dissolved in
439 MeOH and seems to be faster at higher storage temperatures. It should be noted that no
440 significant difference was observed between the aliquots stored at +4°C and room
441 temperature, but this was likely due to the saturation of the detector after the injection of high
442 concentration standard solutions.
443
444 The results obtained from the experiment were in good agreement with what was reported by
445 Tyczkowska *et al.* [53] and Pellicciotti *et al.* [54]. Although no degradation appeared to be
446 observed for the carbapenems and the cephalosporins, MeOH was completely removed from

447 the standard preparation procedure, in order to avoid the presence of this solvent in the mixed
448 standard solutions. As a consequence, alternative solvents (H_2O:MeCN or DMSO) were
449 selected. In addition, the standard solutions were stored in small aliquots (2.5 mL) at -80°C.
450
451 *3.3. Method validation*
452
453 *3.3.1. Identification and confirmatory criteria*
454 In order to satisfy the confirmatory criteria outlined in 2002/657/EC, three identification
455 points (one point for the precursor ion and 1.5 points for each product ion) are required to
456 identify a group B substance. This criteria was exceeded in the proposed method, with four
457 identification points obtained for each analyte. All the retention times and ion ratios measured
458 during the validation study were within the maximum permitted tolerances [42], showing the
459 suitability of the method in the identification and confirmation of 30 β-lactams in bovine
460 muscle samples.
461
462 *3.3.2. Selectivity, linearity, LODs and LOQs*
463 Analytes and internal standards were injected individually in order to monitor for
464 interferences in the UHPLC-MS/MS traces. The effects of cross-talk and isobaric
465 interferences were minimised by identifying unique product ions for each compound and
466 optimising the chromatographic separation, respectively. During the selectivity study, no
467 matrix interferent peaks were observed for the majority of the analytes. An interference was
468 observed for cefazolin, cefadroxil and cefadroxil-D_4 product ions at 323.1, 114.1 and 213.1
469 *m/z*, respectively. As a consequence, alternative product ions of lower intensity were selected.
470 An interference was also observed for doripenem and cefalonium at 274.2 and 337.0 *m/z*,
471 respectively. In the case of cefalonium, only two product ions (152.0 and 337.0 *m/z*) were

generated in low energy CID experiments. In contrast, doripenem gave two additional product ions (112.1 and 318.1 *m/z*) to those selected (342.2 and 274.2 *m/z*), but the choice of these ions would have significantly decreased the sensitivity of the method. In addition, the quantifier and the qualifier ions for both analytes were of similar intensity; consequently it could be concluded that the observed peak was a real matrix interference (and not the actual analyte coming from a drug treatment of the animals), as no peaks were observed for the second ion. It is highlighted that the absolute area count obtained for the interferences was found to be negligible in all the tested blank samples, however further investigation would be needed for doripenem and cefalonium by analysing a larger number of bovine muscle samples.

Linearity was achieved over the calibration range of the method. R^2 was ≥ 0.988 for all the analytes, while individual residuals were in the ± 20% range from the calibration curve. LODs ranged between 0.010 μg kg^{-1} and 4.8 μg kg^{-1}; LOQs ranged from 0.035 μg kg^{-1} to 16.0 μg kg^{-1} (Table 2).

3.3.3. Matrix effects

During the matrix effect study, ion suppression was observed for the majority of the analytes, while biapenem, cefoperazone, cefquinome, imipenem and methicillin showed ion enhancement. The greatest amount of ion suppression and enhancement was observed for amoxicillin and methicillin, respectively (75% and 73%). The use of the 10 internal standards significantly improved the precision of the method (Table 2 and Table 3) and represented a distinguishing feature of the proposed work. With the exception of Berendsen *et al.* [2], none of the previously published methods included such a large number of deuterated standards. However, the available labelled compounds were found not suitable in the quantification of

biapenem, cefoperazone, cefquinome, DCD, imipenem, meropenem, methicillin, piperacillin and ticarcillin. Among the analytes corrected using a labelled compound, mecillinam showed the greatest variability (RSD% = 26.2%). It is highlighted that no MRL is required for mecillinam in bovine species. Cefquinome was the only MRL-substance that showed ion enhancement. The use of an internal standard is particularly important for those analytes for which ion enhancement is observed. In the event of positive samples being found, this matrix effect can potentially lead, indeed, to false non-compliant results. However, in the proposed method, the available labelled compounds were found to be unsuitable for the quantification of cefquinome. In addition, the matrix effect study showed a variability of 28.9%. This problem should be addressed in the future through the implementation of a suitable isotopically labelled standard.

3.3.4. Trueness, precision, absolute recovery, CCα and CCβ

The 2002/657/EC guidelines specify that the trueness for mass fractions between 1 and 10 µg kg^{-1} should be in the range 70-110%, while the trueness for mass fractions ≥ 10 µg kg^{-1} should be in the range 80-110%. The trueness was satisfactory for all the analytes under WLr conditions (range of 86-108%). On day two of the WLR study, the mixed internal standard solution was accidentally added twice to one of the samples fortified at 1.5 MRL/TL, while the internal standard was not added to the following sample. Consequently, six replicates were used on that day for the assessment of WLR at 1.5 MRL/TL for those analytes corrected using an internal standard. The trueness was satisfactory for the majority of substances under WLR conditions (range of 81-108%). The exceptions were mecillinam (69% accuracy at TL), meropenem (115-125%) and faropenem (119-143%). For mecillinam and faropenem, the trueness was outside the acceptable range even though the internal standards cefazolin-$^{13}C_2{}^{15}N$ and ampicillin-D_5 were used, respectively. In the case of meropenem, for which no

internal standards were used, the corresponding labelled compound, namely meropenem-D_6, could be implemented into the method to address the problem. The 2002/657/EC guidelines also state that the coefficient of variations (CVs) for mass fractions between 100 and 1000 µg kg^{-1} shall be ≤ 23% in WLR conditions. For mass fractions lower than 100 µg kg^{-1}, the CVs shall be as low as possible. In addition, the CVs calculated under WLr conditions should not be greater than two thirds of the CVs calculated under WLR conditions. The precision of the method was satisfactory for all analytes in both WLR (2.0-29.9%) and WLr studies (1.5-17.3%), as the CVs did not exceed the maximum CVs calculated by the Horwitz equation and two thirds of these values, respectively.

CCα ranged from 10.6 µg kg^{-1} (cefazolin) to 677 µg kg^{-1} (DCD), depending on the analyte. Overall absolute recoveries (OAR) ranged from 61% to 89% for all the compounds, except for cefadroxil, DCCD, DCD, imipenem and ertapenem (Table 3). Cefadroxil, ertapenem and imipenem are relatively polar analytes, and the lower recovery rates were probably due to the incomplete extraction from the matrix, caused by the specific extraction solvent composition used in the sample preparation. In the case of DCCD and DCD, the disulphide bonds present in their molecular structures likely undergo exchange with protein thiols or other disulphide bonds in the homogenised tissue samples, resulting in losses during the deproteinization step or in the non-detection by the LC-MS/MS system [17]. It is important to state that all active metabolites should be included in the analysis of ceftiofur residues in food products, as the MRL for muscle tissue in all mammalian food-producing species is defined as the sum of all residues retaining the β-lactam structure, expressed as desforylceftiofur [7]. After intramuscular administration, ceftiofur is rapidly metabolised to desfuroylceftiofur and further products, including protein-bound desfuroylceftiofur [26]. Since no hydrolysis is involved, the method described in this study does not detect protein-bound metabolites, consequently it leads to an underestimation of the total ceftiofur content. To measure the total

amount of ceftiofur present in a sample, all the disulphide bonds should be reduced and desfuroylceftiofur released, followed by stabilisation of the thiol group by derivatization [22, 26].

Cephapirin could not be directly included in the proposed method. A very rapid conversion of cephapirin to its active metabolite (DAC) in milk, kidney homogenate and fortified muscle tissue samples was reported in literature [16, 41, 55, 56]. The preliminary experiments performed during the development of the proposed method employed the use of mixes containing both cephapirin and DAC and showed no absolute recovery for cephapirin and 180-190% absolute recovery for DAC. For this reason, a quantitative conversion of cephapirin to DAC was hypothesised after fortification of blank muscle tissue homogenate samples (in approximately 15 min), resulting in the impossibility to quantify cephapirin directly. However, as highlighted by Fagerquist and Lightfield (2003), if this compound was present in a real sample, it would be rapidly and completely converted to DAC after a preliminary homogenisation aimed to obtain a representative sample [16].

Although no MRLs are required for cefacetrile, cefalonium, cefazolin, cefoperazone, mecillinam and penicillin V in bovine muscle, the inclusion of these drugs in the proposed method could provide useful information on their transmission through the food chain in the case that positive samples are found. In addition, as a MRL is required for penicillin V in liver, kidney, skin and fat, and for cefacetrile, cefalonium, cefazolin and cefoperazone in milk, the sample preparation could be adapted to different matrices, while using the same UHPLC-MS/MS method.

4. Comparison with other existing methods

Relatively few multi-residue LC-MS methods have been published for the analysis of β-lactams in muscle tissue. A preliminary literature review found few methods dealing with penicillins and cephalosporins [19-21, 25, 27-40]. Most of these methods include few analytes or exclude cephapirin and ceftiofur metabolites. On the other hand, other methods describe dedicated tests for ceftiofur and its metabolites only [23, 26]. With the exception of Berendsen *et al.* [2], no methods have been reported at all for carbapenems in food products. Although the Berendsen method represents the most valid approach for monitoring ceftiofur and the total amount of its metabolites, it involves a complex derivatisation process followed by SPE clean-up. A time consuming sample preparation including SPE clean-up is also involved in the method reported by Becker *et al.* [41] (Fig. 7). The method described in this study consists of a fast and easy sample preparation protocol and allows the simultaneous analysis of 30 β-lactam antibiotics, including carbapenems, DAC and two of the ceftiofur metabolites, in a reasonably short chromatographic run (18 min). The method published by Geis-Asteggiante *et al.* (2012) had previously applied the same sample preparation to bovine muscle for the analysis of several veterinary drug residues belonging to different classes of antibiotics [50]. It has to be said that the development of a multi-residue method is particularly difficult when several analytes with a wide range of chemical properties are present. However, for the 10 β-lactams included in the Geis-Asteggiante method, the average reproducibility %CV ranged from 8.0% to 49.0%, while the reproducibility of the proposed method could be improved by using 10 different internal standards and ranged between 2.0% and 29.9%. Finally, limits of quantitation for the MRL-substances were well below half the MRLs set by current legislation [7].

5. Conclusions

This research has demonstrated that it is possible to accurately measure 30 β-lactam residues belonging to the penicillin, cephalosporin and carbapenem families in bovine muscle samples by UHPLC-MS/MS following a generic sample preparation protocol. A range of different clean-up procedures were evaluated during method development, and d-SPE with C_{18} was found to give the best overall recoveries. The performance of the method was assessed during matrix effects studies and validation studies, showing it was necessary to include isotopically labelled internal standards to improve accuracy. It was shown in this work that β-lactam residues are particularly unstable and protocols need to be implemented in test methods to mitigate against this problem. Firstly, this work demonstrated that MeOH should not be employed for the preparation of standards, as it causes degradation of some penicillins. This can potentially lead to the identification and selection of incorrect precursor/product ions, as well as an overestimation of beta-lactam residue levels in test samples. This standard stability problem can be addressed through the use of alternative solvents, such as H_2O/MeCN and DMSO. Additionally, it is recommended that MeOH should not be used during sample preparation or during UHPLC-MS/MS analysis. This research concurs with previous research findings that proposed that temperature needs to be carefully controlled during β-lactam analysis. Our study found that analyte recovery and method precision can be improved by extraction over ice, maintaining evaporation at 40°C and reducing chromatographic column temperature to 30°C.

The final method is advantageous over previous methods because it includes the widest range of β-lactams and allows the analysis of carbapenem residues without the need for derivatisation. The method has a reasonably high throughput that allows one analyst working alone to analyse 30 test samples along with calibration and quality control samples in one

day. The method was validated according to the 2002/657/EC guidelines and was demonstrated to be fit for purpose.

Acknowledgements

This research was funded by the FIRM programme administered by the Irish Department of Agriculture, Food and the Marine (Contract 13/F484). We would like to thank Simona Pellicciotti and Guglielmo Dusi for their advice on the stability of standard solutions (Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna, Brescia, Italy).

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Table 1. UHPLC-MS/MS conditions for β-lactam antibiotics.

Analyte	Measured ion	RT (min)	Precursor ion (*m/z*)	Product ions (*m/z*)	Dwell time (s)	CV (V)	CE (eV)	MRM[b]
Imipenem	$[M+H]^+$	2.28	300.1	142.0[a]/126.1	0.194	22	28/17	1
Biapenem	$[M+H]^+$	2.81	351.2	110.2[a]/170.1	0.180	25	19/20	2
Amoxicillin	$[M+H]^+$	3.21	366.1	114.0[a]/349.1	0.035	18	20/9	3
Amoxicillin-D_4	$[M+H]^+$	3.21	370.6	354.0	0.020	16	9	3
Cefadroxil	$[M+H]^+$	3.24	364.1	208.1[a]/158.0	0.030	15	9/9	3
Cefadroxil-D_4	$[M+H]^+$	3.23	368.6	115.0	0.012	16	20	3
DAC	$[M+H]^+$	3.28	382.1	152.1[a]/226.1	0.030	29	24/23	3
DAC-D_6	$[M+H]^+$	3.27	387.9	115.1	0.015	31	45	3
Doripenem	$[M+H]^+$	3.35	421.1	342.2[a]/274.2	0.021	20	13/16	3
Meropenem	$[M+H]^+$	3.50	384.1	141.0[a]/114.1	0.103	23	15/23	4
Ampicillin	$[M+H]^+$	3.75	350.1	106.1[a]/192.1	0.033	22	21/16	5
Ampicillin-D_5	$[M+H]^+$	3.74	355.0	197.1	0.025	18	16	5
Cephalexin	$[M+H]^+$	3.74	348.1	158.1[a]/174.1	0.036	20	9/17	5
Cephalexin-D_5	$[M+H]^+$	3.73	353.0	179.1	0.025	17	14	5
Cephapirin	$[M+H]^+$	3.76	424.0	292.1[a]/152.1	0.040	24	15/24	5
Cefquinome	$[M+H]^+$	4.05	529.0	134.2[a]/167.1	0.074	21	13/23	6
Ertapenem	$[M+H]^+$	4.19	476.1	432.1[a]/233.2	0.016	20	9/14	6
Mecillinam	$[M+H]^+$	4.19	326.1	167.2[a]/139.2	0.022	34	15/29	6
DCCD	$[M+H]^+$	4.26	548.7	183.1[a]/241.2	0.032	36	29/20	6
DCCD-D_3	$[M+H]^+$	4.26	551.9	366.0	0.032	36	21	6
Cefalonium	$[M+H]^+$	4.24	458.9	152.0[a]/337.0	0.006	18	18/10	6
Cefacetrile	$[M+NH_4]^+$	4.92	357.0	280.1[a]/252.1	0.056	16	9/14	7
Cefotaxime	$[M+H]^+$	4.92	456.1	396.0[a]/167.1	0.080	23	10/19	7
Faropenem	$[M+Na]^+$	5.04	308.0	178.0[a]/222.1	0.047	26	18/12	8
Cefazolin	$[M+H]^+$	5.13	454.9	156.0[a]/295.1	0.047	17	15/15	8
Cefazolin-$^{13}C_2{}^{15}N$	$[M+H]^+$	5.13	457.8	298.1	0.047	15	16	8
Cefuroxime	$[M+NH_4]^+$	5.39	442.0	364.1[a]/336.1	0.129	16	14/14	9
Cefoperazone	$[M+H]^+$	5.57	646.0	143.2[a]/530.0	0.022	25	33/12	10
DCD	$[M+2H]^{2+}$	5.71	429.2	183.1[a]/397.0	0.026	23	21/13	10
Ticarcillin	$[M+H]^+$	5.82	385.1	160.1[a]/114.1	0.039	25	14/36	10
Methicillin	$[M+H]^+$	5.98	381.1	165.2[a]/222.2	0.038	27	23/18	10
Piperacillin	$[M+H]^+$	6.02	518.0	143.2[a]/160.1	0.020	28	17/10	10
Penicillin G	$[M+H]^+$	6.23	335.1	160.1[a]/176.1	0.135	21	12/12	11
Penicillin G-D_7	$[M+H]^+$	6.21	342.0	183.1	0.080	18	11	11
Penicillin V	$[M+H]^+$	6.54	351.1	160.1[a]/114.1	0.139	21	13/33	12
Penicillin V-D_5	$[M+H]^+$	6.52	355.9	160.0	0.050	20	15	12
Oxacillin	$[M+H]^+$	6.76	402.1	160.1[a]/243.2	0.150	20	16/14	13
Cloxacillin	$[M+H]^+$	7.04	436.0	160.1[a]/277.1	0.115	22	12/12	14
Nafcillin	$[M+H]^+$	7.16	415.1	199.2[a]/171.2	0.081	20	7/28	14
Nafcillin-D_5	$[M+H]^+$	7.15	420.0	261.1	0.050	19	17	14
Dicloxacillin	$[M+H]^+$	7.41	470.0	160.2[a]/114.2	0.151	22	14/34	15

a = Quantitation ion; RT = Retention Time; CV = Cone Voltage; CE = Collision Energy.

b = MRM Windows: 1 = (1.99 – 2.98 min); 2 = (2.60 - 3.13 min); 3 = (2.98 – 3.50 min); 4= (3.41 – 3.62 min); 5 = (3.60 – 3.88 min); 6 = (3.91 – 4.42 min); 7 = (4.75 – 5.10 min); 8 = (4.93 – 5.26 min); 9 = (5.23 – 5.57 min); 10 = (5.35 – 6.27 min); 11 = (6.05 – 6.36 min); 12 = (6.36 – 6.69 min); 13 = (6.62 – 6.99 min); 14 = (6.82 – 7.35 min); 15 = (7.23 – 7.58 min).

Table 2. Calibration ranges, LODs, LOQs and matrix effect data. Positive values indicate ion suppression, while negative values indicate matrix enhancement.

Analyte	Calibration range (µg kg^{-1})	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	ME%[a]	RSD(%) (no IS)	RSD(%) (IS)
Amoxicillin	12.5-100	0.994	0.50	1.7	75	17.2	4.6
Ampicillin	12.5-100	0.994	0.050	0.17	34.1	12.2	3.2
Cloxacillin	75-600	0.998	0.15	0.50	13.8	4.7	4.5
Dicloxacillin	75-600	0.998	0.10	0.30	10.6	6.7	5.5
Mecillinam	2.5-20	0.996	0.030	0.10	19.7	27.0	26.2
Methicillin	2.5-20	0.994	0.030	0.10	-73	19.6	No IS used
Nafcillin	75-600	0.998	0.030	0.10	9.5	4.2	1.6
Oxacillin	75-600	0.992	0.010	0.035	17.0	5.4	4.9
Penicillin G	12.5-100	0.993	0.040	0.12	19.2	7.2	4.1
Penicillin V	6.25-50	0.998	0.020	0.070	14.3	3.6	3.7
Piperacillin	2.5-20	0.997	0.040	0.12	5.8	9.3	No IS used
Ticarcillin	5-40	0.993	0.30	1.0	21.7	10.0	No IS used
Cefacetrile	5-40	0.994	0.30	1.0	44.6	22.7	12.5
Cefadroxil	12.5-100	0.988	1.3	4.2	69	22.0	11.0
Cephalexin	50-400	0.998	0.50	1.7	23.7	10.9	3.2
Cefalonium	5-40	0.989	0.30	1.0	42.0	20.2	13.9
Cefazolin	2.5-20	0.997	0.10	0.30	35.8	13.8	3.7
Cefoperazone	5-40	0.994	0.30	1.0	-15.2	9.2	No IS used
Cefotaxime	5-40	0.994	0.30	1.0	43.3	24.6	14.1
Cefquinome	12.5-100	0.989	0.070	0.22	-34.1	28.9	No IS used
Cefuroxime	5-40	0.995	0.30	1.0	6.8	7.8	7.7
DAC	12.5-100	0.995	0.60	1.9	70	23.6	5.8
DCCD	125-1000	0.993	4.8	16.0	44.1	14.3	4.6
DCD	125-1000	0.990	0.20	0.70	52	10.1	No IS used
Biapenem	5-40	0.992	0.30	1.0	-34.8	19.5	No IS used
Doripenem	12.5-100	0.991	1.0	3.0	42.9	25.0	21.6
Ertapenem	50-400	0.994	1.5	5.0	30.5	8.6	8.7
Imipenem	12.5-100	0.994	0.50	1.7	-45.8	15.2	No IS used
Meropenem	5-40	0.997	0.30	1.0	61	12.3	No IS used
Faropenem	12.5-100	0.995	0.50	1.7	48.1	35.8	25.0

a = Results are based on the analysis of 27 different bovine muscle samples; IS = Internal Standard.

Table 3. Validation results for bovine muscle.

Analyte	MRL/TL (µg kg^{-1})	Trueness% (WLr%)			Trueness% (WLR%)			CCα (µg kg^{-1})	CCβ (µg kg^{-1})	OAR% (RSD%, n = 3)	
		0.5 MRL/TL	MRL/TL	1.5 MRL/TL	0.5 MRL/TL	MRL/TL	1.5 MRL/TL			2LCL	0.875HCL
Amoxicillin	50	102 (5.9)	101 (4.5)	103 (5.2)	101 (6.4)	101 (5.4)	101 (5.8)	55	60	69 (2.4)	70 (5.5)
Ampicillin	50	101 (2.4)	99 (2.4)	101 (3.3)	100 (4.3)	102 (3.6)	100 (4.0)	54	58	78 (7.9)	76 (4.8)
Cloxacillin	300	103 (2.2)	101 (2.2)	101 (1.7)	102 (6.0)	99 (5.2)	95 (7.5)	350	399	81 (5.6)	88 (3.6)
Dicloxacillin	300	104 (2.4)	103 (2.3)	104 (5.0)	102 (6.2)	99 (3.7)	97 (5.6)	321	351	83 (2.3)	87 (3.4)
Mecillinam	10	96 (2.5)	97 (3.3)	100 (4.0)	81 (29.0)	69 (29.9)	91 (21.6)	13.1	19.6	85 (1.1)	83 (2.5)
Methicillin	10	108 (10.2)	104 (9.5)	98 (6.7)	91 (13.2)	90 (14.8)	91 (13.0)	12.2	15.8	85 (5.0)	84 (4.4)
Nafcillin	300	101 (2.2)	101 (1.8)	100 (1.5)	104 (6.2)	101 (3.2)	99 (4.2)	326	350	84 (2.0)	88 (4.2)
Oxacillin	300	103 (2.7)	101 (3.2)	101 (3.2)	105 (6.4)	103 (3.6)	101 (4.3)	322	347	78 (12.6)	89 (5.8)
Penicillin G	50	106 (2.8)	105 (2.8)	105 (2.4)	100 (6.7)	104 (12.6)	103 (14.9)	62	84	79 (12.5)	85 (3.1)
Penicillin V	25	101 (2.4)	100 (2.3)	102 (2.8)	98 (4.8)	98 (2.3)	100 (2.8)	26.0	27.3	87 (1.5)	86 (3.4)
Piperacillin	10	104 (4.9)	104 (5.8)	105 (5.3)	102 (7.4)	102 (6.1)	102 (5.3)	11.5	12.6	89 (4.0)	85 (4.6)
Ticarcillin	20	99 (6.4)	99 (8.6)	100 (6.7)	99 (10.0)	105 (10.3)	95 (8.9)	28.5	34.1	61 (5.3)	62 (6.6)
Cefacetrile	20	101 (4.3)	100 (4.6)	99 (8.2)	108 (9.6)	104 (5.5)	101 (8.9)	22.9	27.1	80 (2.7)	79 (3.4)
Cefadroxil	50	100 (5.7)	98 (5.4)	101 (6.3)	101 (10.9)	98 (5.6)	97 (6.7)	54	60	58 (2.1)	61 (8.1)
Cephalexin	200	98 (1.9)	98 (1.9)	100 (3.5)	101 (3.5)	100 (2.0)	99 (3.6)	202	213	69 (4.8)	67 (3.9)
Cefalonium	20	100 (8.9)	100 (5.6)	100 (6.0)	99 (12.6)	98 (8.0)	101 (8.5)	21.9	25.0	79 (13.0)	81 (2.4)
Cefazolin	10	101 (5.0)	101 (4.5)	100 (5.2)	98 (6.8)	101 (5.6)	101 (8.3)	10.6	11.9	81 (4.6)	83 (4.2)
Cefoperazone	20	104 (5.5)	101 (7.0)	102 (6.3)	101 (6.7)	100 (8.5)	101 (8.0)	23.7	27.4	86 (3.7)	86 (6.7)
Cefotaxime	20	102 (6.4)	104 (5.2)	101 (6.5)	101 (6.6)	104 (6.1)	107 (8.8)	21.9	25.0	64 (5.0)	67 (7.0)
Cefquinome	50	103 (7.0)	101 (11.5)	100 (6.9)	95 (10.5)	96 (12.7)	91 (7.1)	68	79	87 (3.5)	86 (5.3)
Cefuroxime	20	104 (7.2)	102 (6.7)	101 (7.2)	100 (7.2)	105 (6.7)	104 (10.7)	21.1	24.7	87 (1.4)	84 (3.2)
DAC	50	102 (4.9)	101 (4.4)	101 (3.7)	99 (6.8)	97 (5.2)	99 (4.1)	53	57	77 (5.0)	78 (2.7)
DCCD	500[a]	104 (4.8)	101 (5.9)	104 (4.7)	101 (4.8)	99 (5.9)	99 (4.7)	536	584	40.3 (7.8)	45.1 (2.9)
DCD	500[a]	108 (5.2)	102 (7.0)	103 (8.8)	99 (9.7)	106 (9.4)	98 (8.8)	677	802	53 (4.7)	56 (7.9)
Biapenem	20	106 (5.6)	103 (2.9)	103 (5.8)	91 (14.2)	84 (13.9)	92 (8.2)	23.0	27.3	71 (2.2)	71 (3.6)
Doripenem	50	106 (4.8)	103 (7.1)	100 (4.7)	96 (10.9)	94 (8.8)	96 (12.3)	57	71	62 (3.9)	65 (2.8)
Ertapenem	200	101 (4.7)	97 (4.3)	102 (8.0)	96 (9.0)	95 (7.3)	100 (8.4)	213	242	44.2 (6.4)	43.3 (6.8)
Imipenem	50	106 (6.0)	100 (8.4)	86 (10.9)	96 (10.9)	102 (10.2)	86 (13.9)	69	90	47.0 (8.3)	51 (4.9)

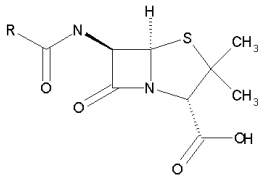
Table 3. *(Continued)*

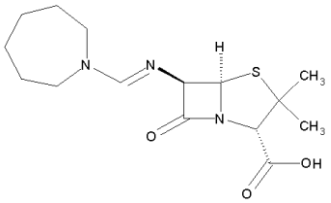
Analyte	MRL/TL (µg kg^{-1})	Trueness% (WLr%)			Trueness% (WLR%)			CCα (µg kg^{-1})	CCβ (µg kg^{-1})	OAR% (RSD%, n = 3)	
		0.5 MRL/TL	MRL/TL	1.5 MRL/TL	0.5 MRL/TL	MRL/TL	1.5 MRL/TL			2LCL	0.875HCL
Meropenem	20	104 (3.6)	100 (6.0)	99 (3.6)	115 (21.5)	125 (23.4)	122 (22.4)	28.4	44.1	66 (0.46)	66 (8.4)
Faropenem	50	97 (17.3)	96 (14.8)	97 (11.7)	128 (17.8)	143 (14.9)	119 (13.5)	77	100	78 (0.10)	79 (3.3)

TL = Target Level; OAR = Overall Absolute Recovery; LCL = Lowest Calibration Level; HCL = Highest Calibration Level.

a = Validation levels were 250, 500 and 750 µg kg^{-1} (MRL established by (EU) 37/2010 is 1000 µg kg^{-1} for the sum of all residues retaining the β-lactam structure, expressed as desfuroylceftiofur).

Penicillins

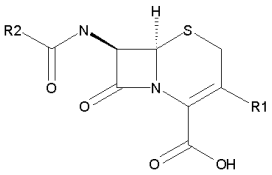


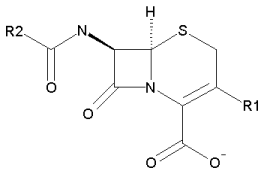


Mecillinam

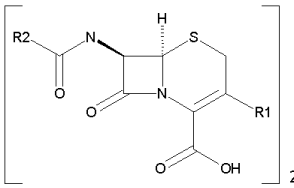
Penicillin	*R*	*Penicillin*	*R*
Amoxicillin	-C_7H_8NO	Oxacillin	-$C_{10}H_8NO$
Ampicillin	-C_7H_8N	Penicillin G	-C_7H_7
Cloxacillin	-$C_{10}H_7ClNO$	Penicillin V	-C_7H_7O
Dicloxacillin	-$C_{10}H_6Cl_2NO$	Piperacillin	-$C_{14}H_{16}N_3O_3$
Methicillin	-$C_8H_9O_2$	Ticarcillin	-$C_6H_5O_2S$
Nafcillin	-$C_{12}H_{11}O$		

Cephalosporins





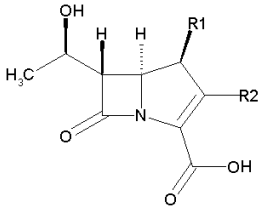
Cefalonium and Cefquinome

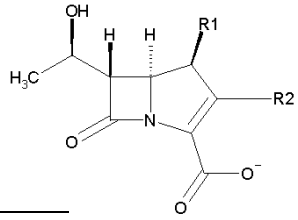


DCD

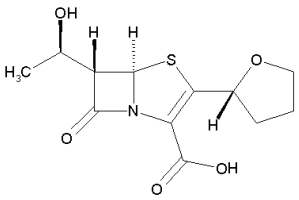
Cephalosporin	*R1*	*R2*	*Cephalosporin*	*R1*	*R2*
Cefacetrile	-$C_3H_5O_2$	-C_2H_2N	Cefotaxime	-$C_3H_5O_2$	-$C_5H_6N_3OS$
Cefadroxil	-CH_3	-C_7H_8NO	Cefquinome	-$C_{10}H_{13}N$	-$C_5H_6N_3OS$
Cephalexin	-CH_3	-C_7H_8N	Cefuroxime	-$C_2H_4NO_2$	-$C_6H_6NO_2$
Cefalonium	-$C_7H_8N_2O$	-C_5H_5S	DAC	-CH_3O	-C_6H_6NS
Cefazolin	-$C_4H_5N_2S_2$	-$C_2H_3N_4$	DCD	-CH_2S	-$C_5H_6N_3OS$
Cefoperazone	-$C_3H_5N_4S$	-$C_{14}H_{16}N_3O_4$	DCCD	-$C_4H_8NO_2S_2$	-$C_5H_6N_3OS$

Carbapenems and faropenem





Biapenem



Faropenem

Carbapenem	*R1*	*R2*
Biapenem	-CH_3	-$C_5H_7N_3S$
Doripenem	-CH_3	-$C_5H_{12}N_3O_2S_2$
Ertapenem	-CH_3	-$C_{12}H_{13}N_2O_3S$
Imipenem	-H	-$C_3H_7N_2S$
Meropenem	-CH_3	-$C_7H_{13}N_2OS$

Fig. 1. Chemical structures of the β-lactams included in the proposed UHPLC-MS/MS method.

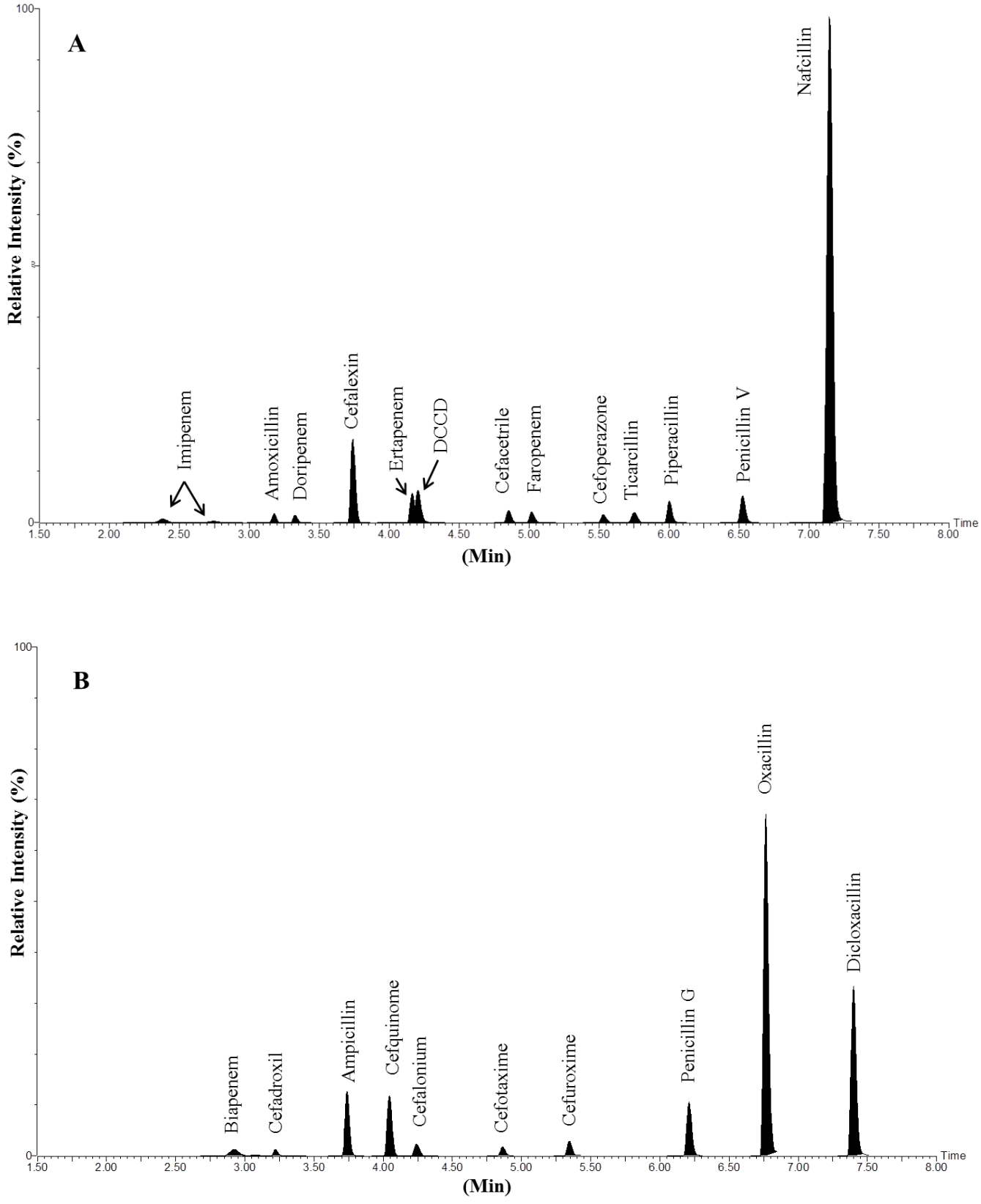
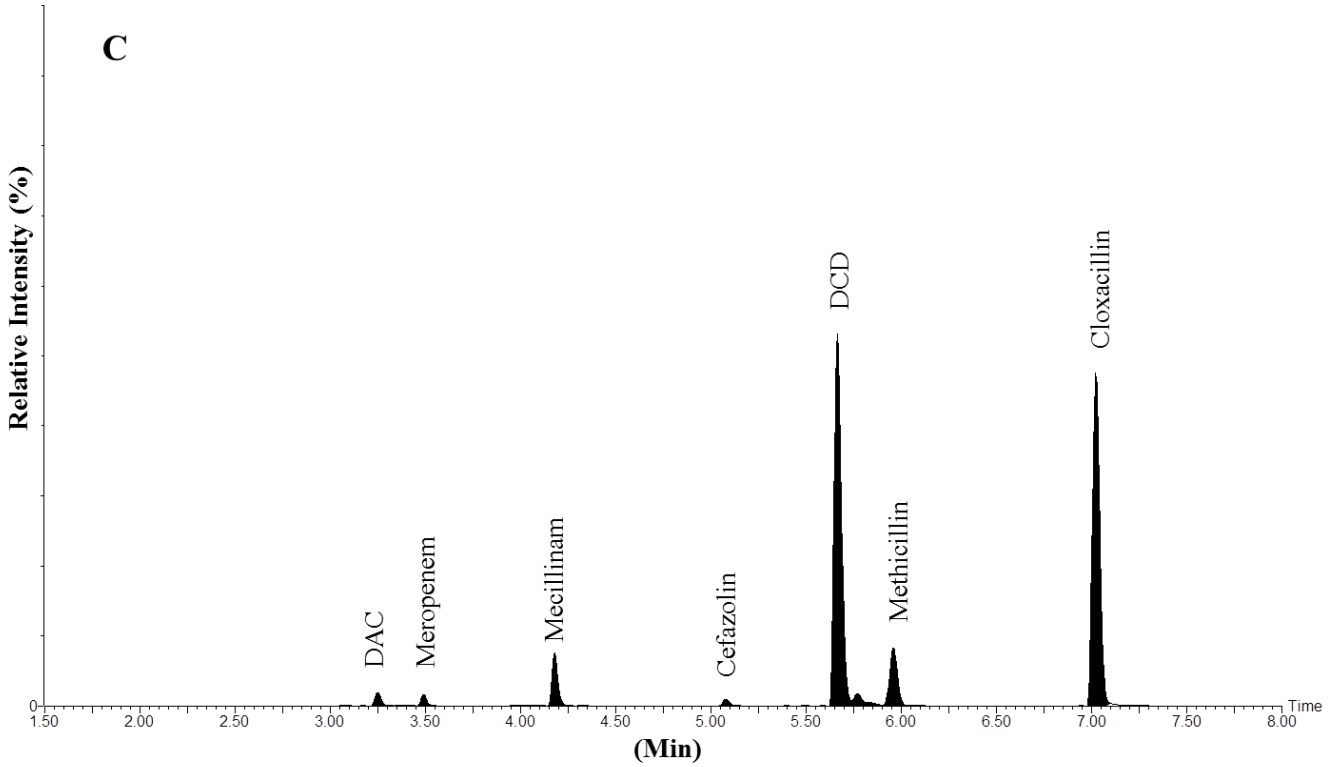


Fig. 2. Overlay of LC-MS/MS chromatograms for the β-lactams (A-C) and internal standards (D-E) included in the method in a blank bovine muscle sample fortified at half the MRL/TL.



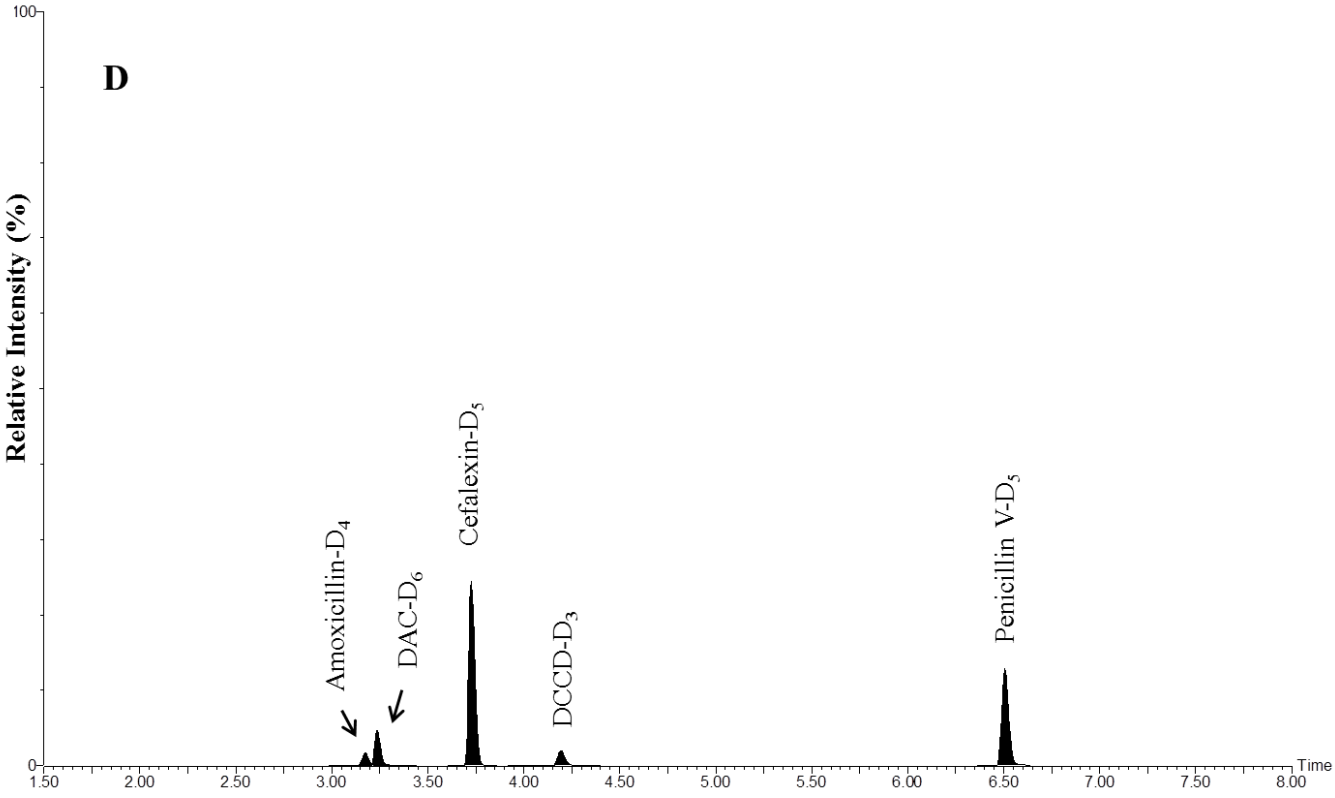


Fig. 2. *(Continued)*

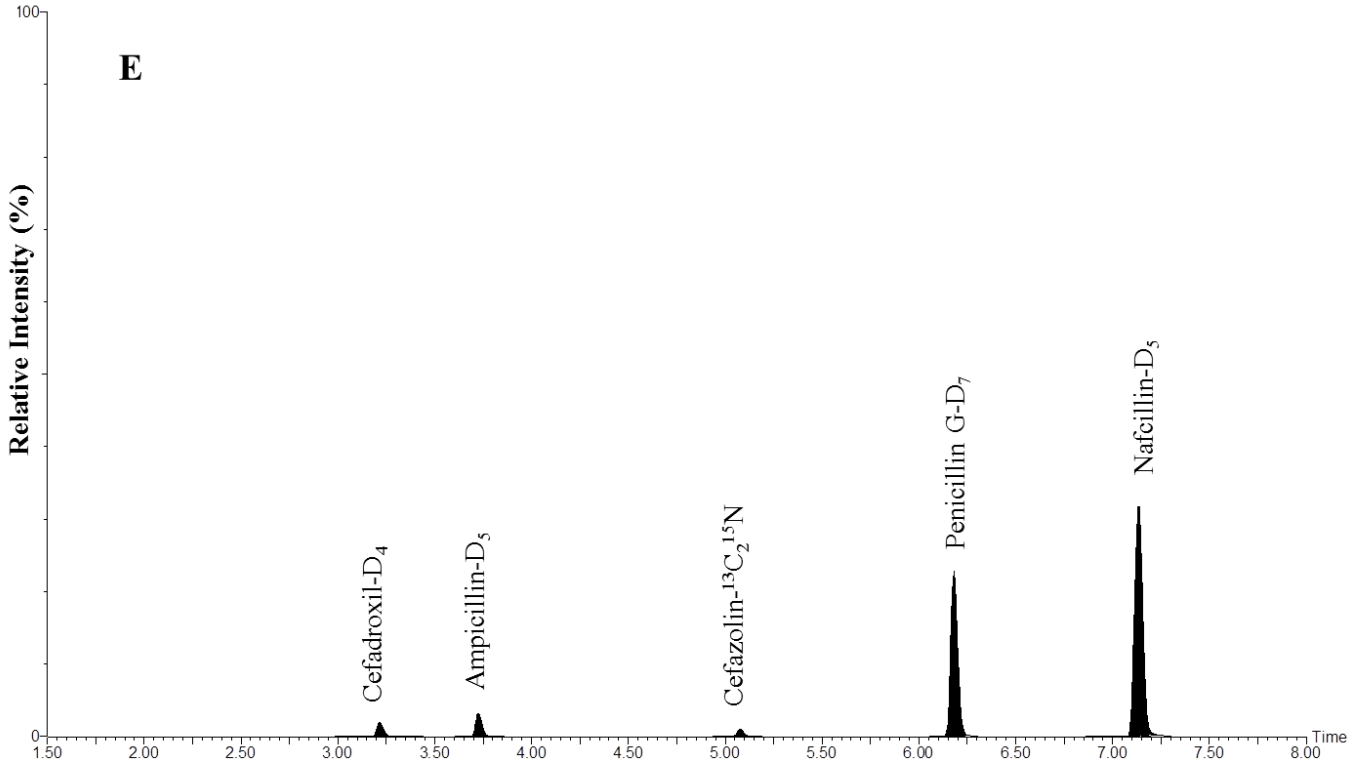


Fig. 2. *(Continued)*

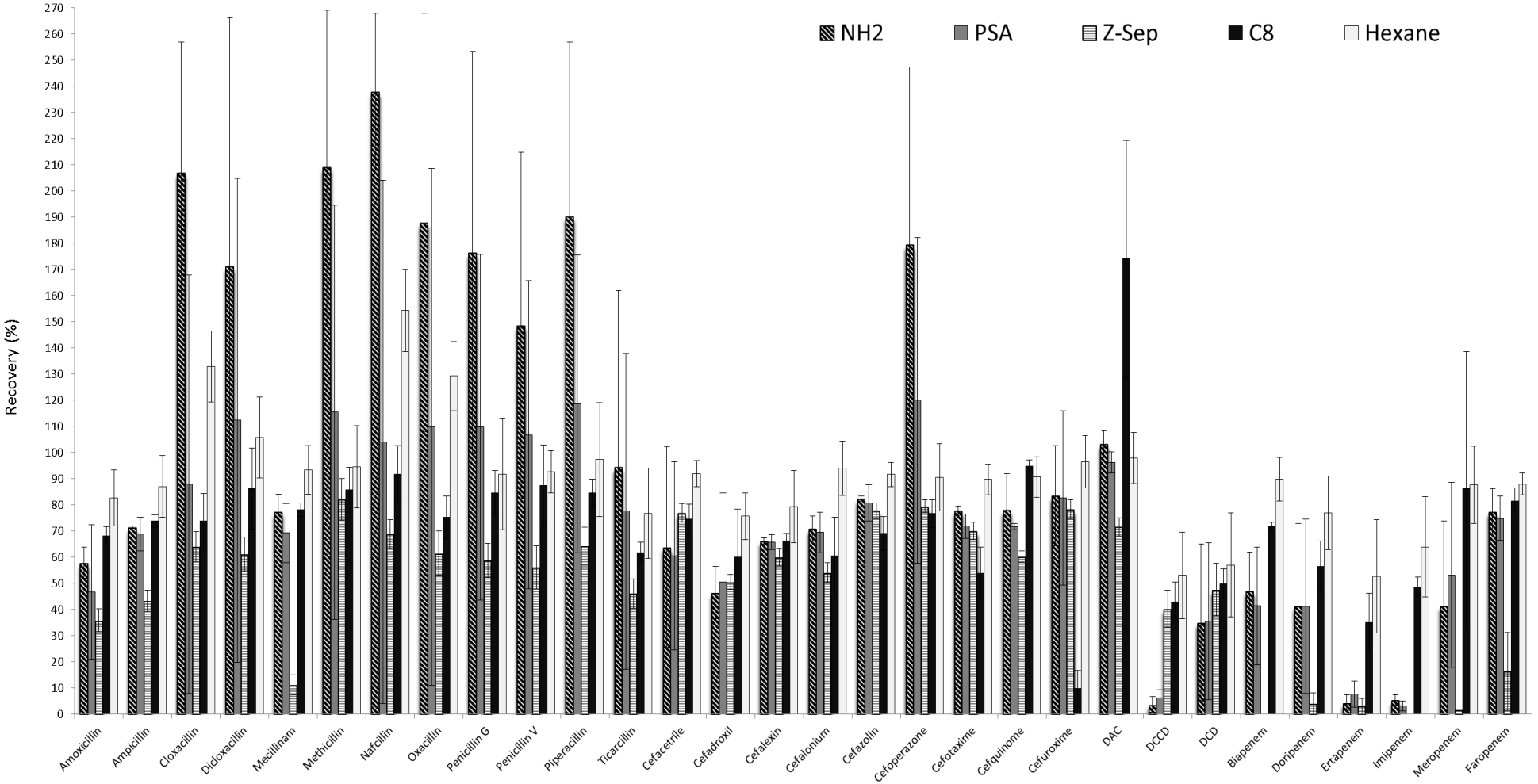


Fig. 3. Average recoveries (and standard deviations, shown by error bars) obtained using NH_2, PSA, Z-Sep and C_8 sorbents or hexane liquid-liquid partitioning clean-up (n = 3).

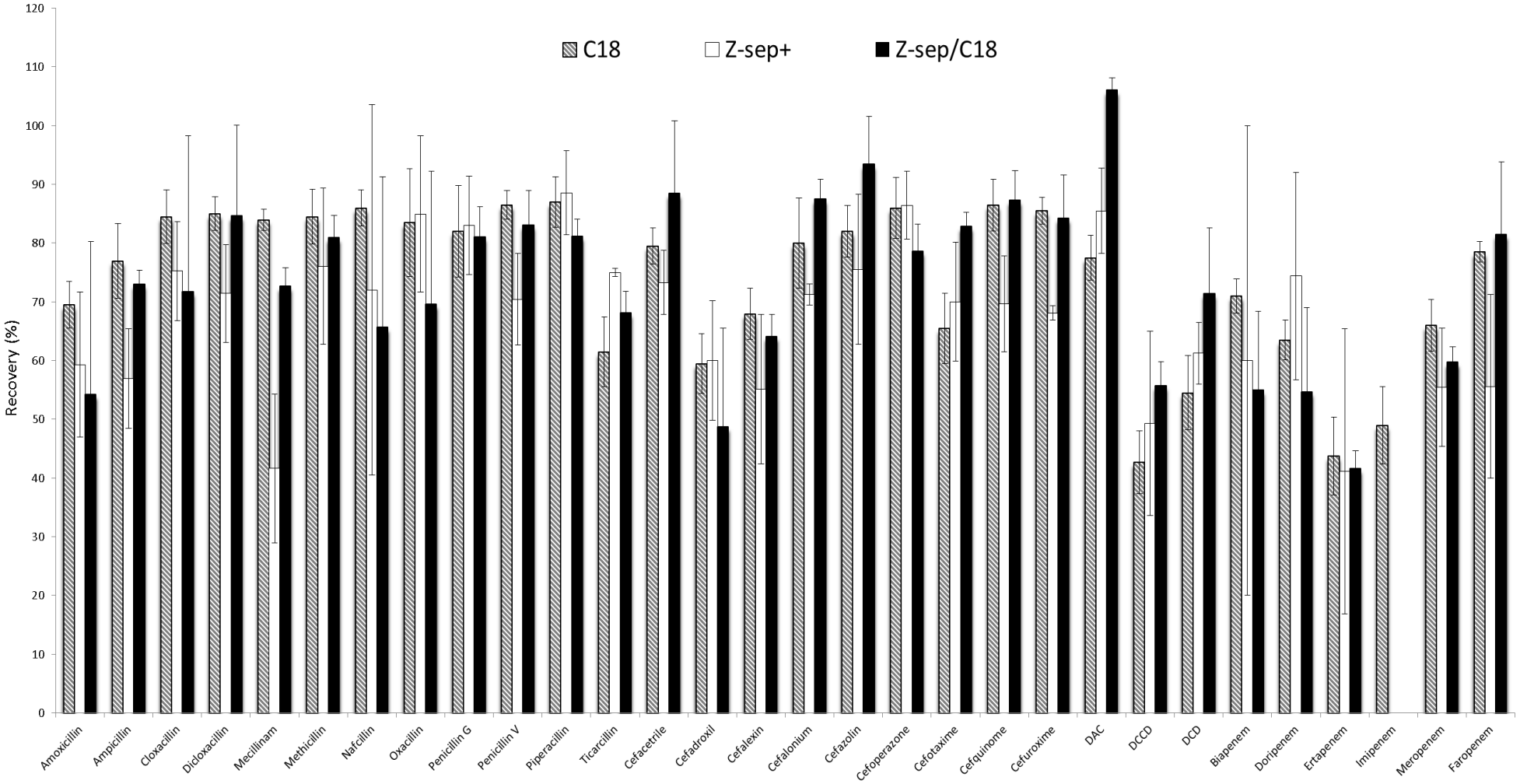


Fig. 4. Average recoveries (and standard deviations, shown by error bars) obtained using C_{18}, Z-Sep+ and Z-Sep/C_{18} sorbents (n = 3).

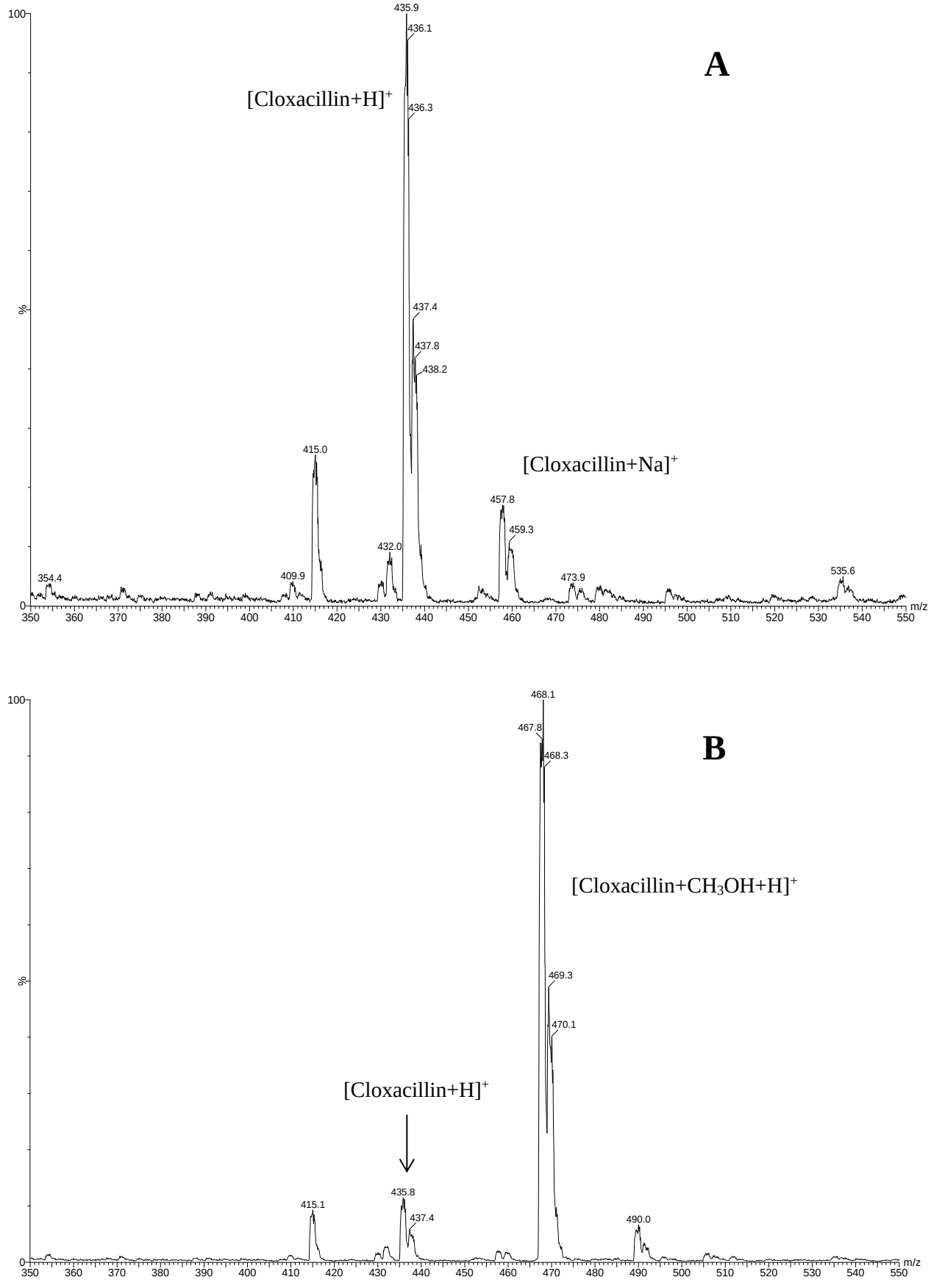


Fig. 5. MS spectra obtained from a cloxacillin standard solution prepared in H_2O:MeCN (A) and a cloxacillin standard solution prepared in MeOH (B), after 11 days of storage at +4ºC.

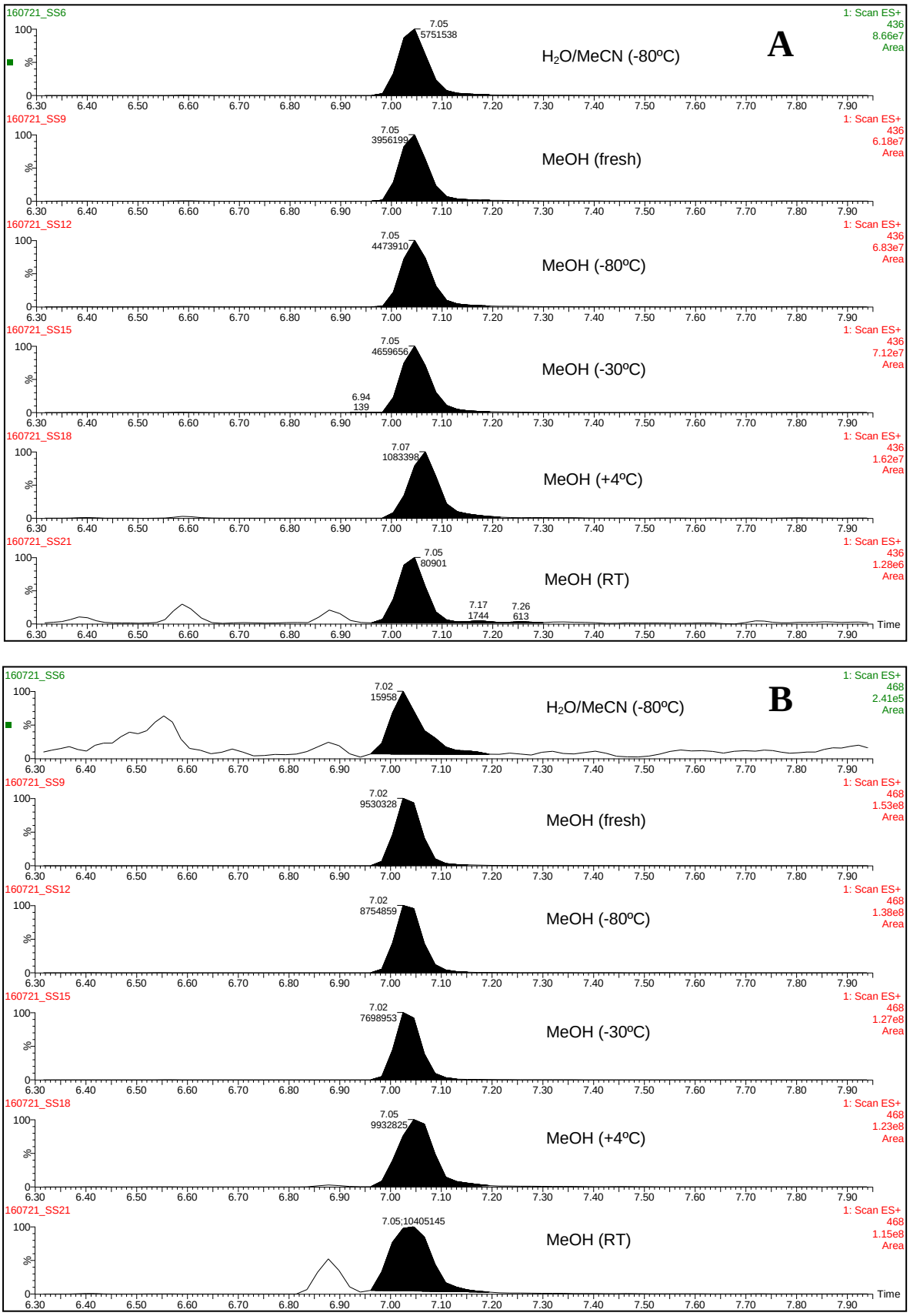


Fig. 6. Extracted chromatograms for the masses 436.0 *m/z* (A) and 468.0 *m/z* (B) obtained after column injections of 10 µg mL^{-1} cloxacillin standard solutions prepared in H_2O:MeCN or MeOH and stored for 11 days at different temperatures. RT = Room Temperature.

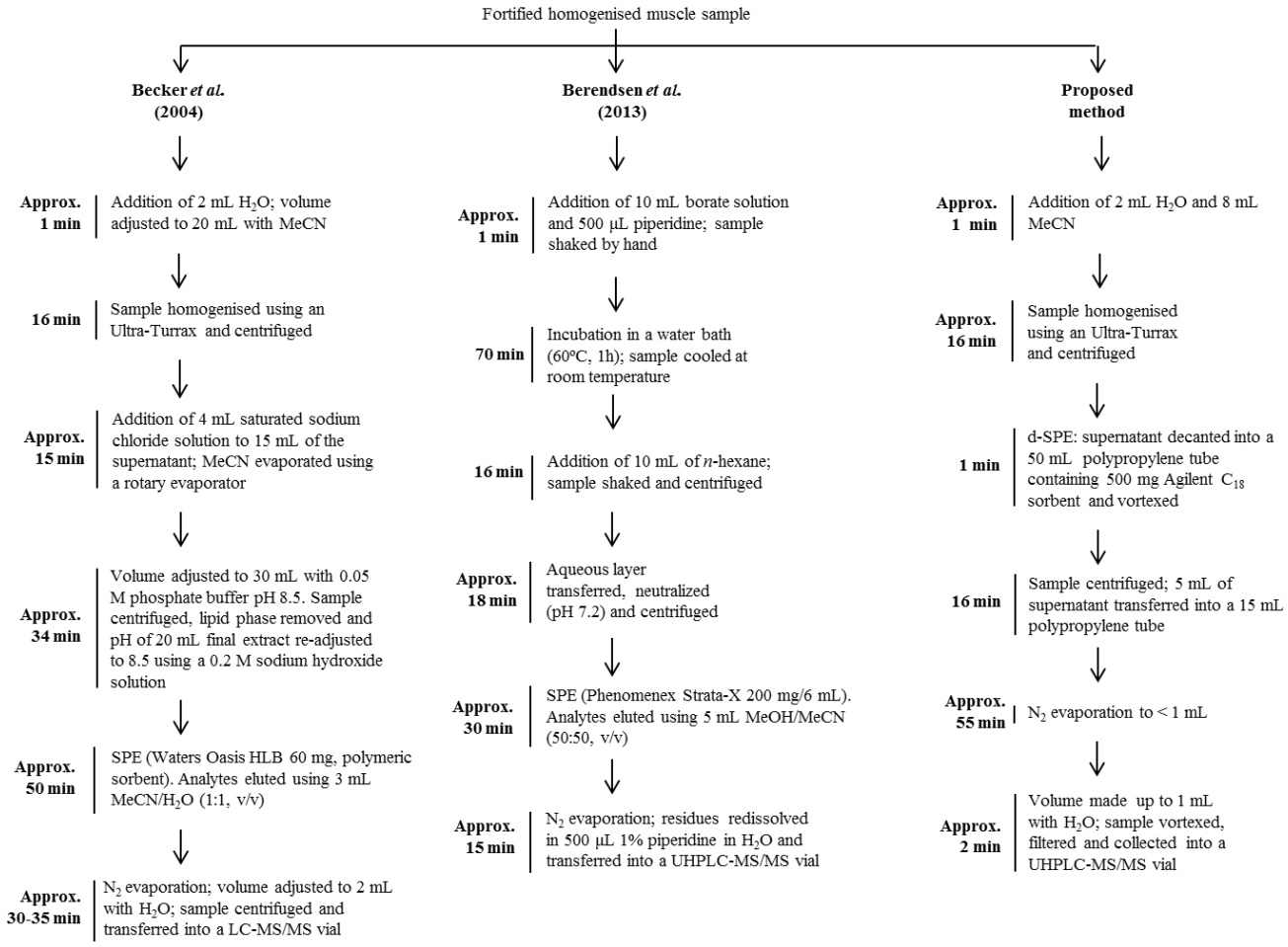


Fig. 7. Flow diagram of published methods (Becker *et al.* (2004) [41]; Berendsen *et al.* (2013) [2]) sample preparation procedures and comparison with the proposed method for the extraction of β-lactam residues from muscle tissue samples, showing steps and time required. The estimated time for each step is based on the extraction of one sample only.